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Transfection and expression of human O^6-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: the role of MGMT in protection against the genotoxic effects of alkylating agents

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O^6-Methylguanine-DNA methyltransferase (MGMT) is responsible for removal of O^6-alkylguanine from DNA induced by alkylating mutagens/carcinogens. To analyze the involvement of O^6-alkylguanine in the generation and MGMT in avoidance of various genotoxic effects of alkylating agents, we transfected Chinese hamster ovary (CHO) cells that lack MGMT activity with human MGMT cDNA cloned into a mammalian expression vector (*pSV2MGMT*). A high proportion (60–80%) of transfectants selected for a co-transfected *neo* gene survived treatment with high doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-hydroxyethyl-*N*-chloroethylnitrosourea (HeCNU). Parallel transfections with an expression vector containing the bacterial *ada* gene (*pSV2ada*) showed the human MGMT to be more effective than the *ada* expression vector in mediating alkylation resistance. Various clonal CHO cell lines have been established stably transfected with the human MGMT cDNA. The transfectants expressed human MGMT at levels ranging from 8600 to 210 000 molecules per cell. The high MGMT expressors became strongly resistant to the killing effects of MNNG, HeCNU, *N*-methyl-*N*-nitrosourea (MNU) and, to a significant lesser degree, methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS). No killing resistance was observed to *N*-ethyl-*N*-nitrosourea (ENU), though the MGMT and *ada* transfectants showed reduction in mutation frequency induced by this agent. Protection from mutation induction by MGMT (and *ada*) expression was also demonstrated for MNNG. The transfectants were also protected from the sister chromatid exchange (SCE) inducing and, to a lesser degree, clastogenic effect of MNNG and MNU, and slightly to EMS and MMS. Again no protection was observed towards ENU. Correlations between MGMT activity and resistance to a given end point suggest that, for MNNG, O^6-methylguanine is the preponderant toxic, mutagenic and SCE inducing lesion. About 90% of MNNG (and MNU) induced SCEs and nearly all of the MNNG-induced gene mutations seem to be due to this adduct. For alkylation-induced chromosomal aberrations, however, and for cell killing and SCEs induced by MMS, EMS and ENU, other lesions than O^6-alkylguanine appear to be of major importance. The data strongly support the view that O^6-methylguanine is a genotoxic lesion and MGMT a function decisively involved in avoidance of genotoxic effects in cells exposed to MNNG and related compounds. They indicate also that it is important to take into account the property and mode of action of any given alkylating agent in assessing the protective role of MGMT against alkylation-induced genotoxicity.

Introduction

Monofunctional alkylating agents are versatile environmental mutagens and carcinogens (1). Once formed or activated they react with DNA at various nucleophilic sites generating more than a dozen different DNA adducts (2). The relative amount of alkyl adducts varies with the type of the agent; it is largely determined by its electrophility (for review see ref. 3). The fate and biological consequences of only some of the induced alkylation lesions are known. It is well established that, among the adducts, O^6-alkyl (methyl and ethyl) guanine is a mutagenic lesion because of its mispairing properties (4–6). O^4-alkyl-thymine also causes mispairing (7), but because it is of much lower relative amount, O^6-alkylguanine seems to be the predominant premutagenic lesion induced by monofunctional alkylating agents. This lesion also appears to be involved in tumor induction (8–12).

O^6-alkylguanine is removed from DNA by O^6-methylguanine-DNA methyltransferase (MGMT*) (13–16). The repair protein transfers the alkyl group to an internal cystein residue. Thereby guanine in DNA is restored and MGMT becomes inactivated (14,17). Because of the stoichiometry of the reaction the repair capacity of the cell for O^6-alkylguanine depends on the level of active MGMT molecules per cell.

The expression of MGMT appears to be regulated in a cell- and tissue-type-specific manner. Significant variation in the level of MGMT was found in mammalian cell lines *in vitro* and in different cell types *in vivo* (18–21). Several established rodent (e.g. V79 and CHO) and human (e.g. HeLa M) cell lines and ~20% of human tumor cells have no detectable MGMT (Mex^- phenotype) (22–24). For MGMT-positive (Mex^+) cells the MGMT level varies between 10^3 and 2×10^5 molecules per cell (19,24,25). In rodent hepatoma cell lines the MGMT gene can be induced by genotoxic stress (26).

O^6-Methylguanine, unlike some *N*-alkylated purines (3-methyladenine and 3-methylguanine), does not block DNA replication (27). In *Escherichia coli* it is not the main toxic lesion (28). For mammalian cells there is some controversy about the nature of alkylation lesion(s) that give rise to cell killing and the induction of sister chromatid exchanges (SCEs) and chromosomal aberrations. It has been reported that SCEs are not related to gene mutations but rather to the toxicity of a given compound (29). For chromosomal aberrations, a correlation between the potential for *N*-alkylation of a given alkylating agent and its clastogenic efficacy has been demonstrated, leading to the conclusion that *N*-alkylations are predominantly involved in chromosomal aberration formation (30). Until now, the most striking evidence for involvement of O^6-methylguanine not only in mutagenesis but also in cell killing and SCE induction derives from work with

*Abbreviations: MGMT, O^6-methylguanine-DNA methyltransferase; Mex^-, MGMT-deficient phenotype; HGPRT, hypoxanthine-guanine phosphoribosyl-transferase; SCE, sister chromatid exchange; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; ENU, *N*-ethyl-*N*-nitrosourea; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; HeCNU, *N*-hydroxyethyl-*N*-chloroethyl-nitrosourea; ssDNA, salmon sperm DNA.

transgenic cell lines. Mex$^-$ HeLa and Chinese hamster cells transfected with the bacterial *ada* gene acquired resistance to the toxic, mutagenic and SCE inducing effects of MNNG and related compounds (31–35). In a preliminary report we showed that *ada* transfectants also become resistant to the clastogenic effect of MNNG (36).

It is questionable, however, whether results obtained in transfection experiments with the *ada* gene are representative for mammalian cells. This is because (i) the Ada protein repairs both O^6-methylguanine, O^4-methylthymine and the *S*-stereoisomer of methylphosphotriesters (37–39). It is therefore difficult, by transfecting *ada* or even the truncated version of *ada* (40–42), to assess the contribution of O^6-alkylguanine in the generation of various genotoxic effects. (ii) The human *MGMT* appears to repair O^6-methylguanine more slowly than the Ada protein. Furthermore, the bacterial protein repairs O^6-ethylguanine less efficiently than the mammalian protein (43,44). (iii) Ada is considerably larger than the mammalian MGMT [38 kDa as compared to 22 kDa for the human MGMT (7,43,45)]. This may hinder its access to lesions in the chromatin and therefore alter the repair efficiency. The most convenient experimental system analyzing the contribution of O^6-alkylguanine in induction of, and the role of mammalian MGMT in protection against genotoxic effects of alkylating agents, is probably provided by isogenic cell lines differing only in MGMT expression. The human MGMT cDNA has recently been cloned (46–48). Using the complete cDNA sequence cloned by Tano *et al.* (46), we have constructed a MGMT expression vector and used it for complementation of the Mex$^-$ phenotype of Chinese hamster cells. By analyzing several isogenic transfectant strains differing only in their MGMT levels we explored in detail the role of MGMT in protection from, and O^6-alkylguanine in induction of cell killing, mutagenesis, SCEs and chromosomal aberrations. Futhermore, we compared the protective effect of the human MGMT with that of the Ada protein in this cell system. Here we show that MGMT provides strong protection to the genotoxic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanine (MNNG) and related compounds. The data point also to remarkable agent- and dose-specific effects, and indicate that the contribution of O^6-alkylguanine to genotoxicity must be considered in the context of other lesions induced by a given alkylating agent.

Materials and methods

Chemicals

N-Methyl-*N*-nitrosourea (MNU) and *N*-ethyl-*N*-nitrosourea (ENU) (Serva) were recrystallized before use. These agents and MNNG (Sigma) were dissolved in a small amount of DMSO and diluted with sterile distilled water. Methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) (Sigma), *N*-hydroxyethyl-*N*-chloroethyl-nitrosourea (HeCNU) (generous gift of Dr Eisenbrandt, Kaiserslautern), G418 (Gibco) and 5-bromodeoxyuridine (Serva) were dissolved in distilled water. 6-Thioguanine (Sigma) was solubilized in 0.1 N NaOH. The mutagen stock solutions were stored in batches at −80°C for up to 2 weeks; they proved to be stable during this period (49). The other stock solutions were kept frozen at −20°C.

Cell culture

CHO-9 cells (50) were cultivated in F12/Dulbecco's MEM (1:1) containing 10% inactivated fetal calf serum (Gibco) and 30 μg/ml gentamycin (Gibco) in a humidified atmosphere containing 6% CO_2. Cells were passaged twice per week. Transfectants were routinely cultivated in medium containing 1.5 mg/ml G418.

Vector construction

The *E.coli ada* gene cloned into pUC9 (the plasmid pYN3059 was a generous gift of Dr Sekiguchi; ref. 51) and the human MGMT cDNA cloned into pUC9 (plasmid pKT100; ref. 46) were recloned into *pSV2neo* and thereby brought under the control of the early SV40 promoter. In brief, the *ada* gene was excised from plasmid pYN3059 by *Hin*dIII and *Sma*I and ligated to the *pSV2neo* vector from which the *neo* gene was removed by *Hin*dIII–*Sma*I digestion. For recloning of MGMT, the cDNA was excised from pKT100 by *Eco*RI, blunt ended and inserted into the blunt ended *pSV2neo* vector with the prior removal of the *neo* gene by *Hin*dIII–*Sma*I. The resulting plasmids were named *pSV2ada* and *pSV2MGMT* (Figure 1).

Transfection and selection procedures

Cells were transfected using a slightly modified calcium phosphate precipitation method (50). Briefly, 5×10^5 cells were seeded per 10 cm dish (10 ml medium) and treated 1 day later with each 20 μg of DNA: 5 μg *pSV2neo* + 15 μg salmon sperm (ss) DNA or 5 μg *pSV2neo* + 5 μg *pSV2ada* + 10 μg ssDNA or 5 μg *pSV2neo* + 5 μg *pSV2MGMT* + 10 μg ssDNA were added per plate. The DNA was dissolved in 280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.1. After dropwise addition of the same volume of 250 mM $CaCl_2$, 10 mM HEPES, pH 7.1, the precipitate was allowed to form during a 60 min incubation period at room temperature. One milliliter of the precipitate (containing 20 μg DNA) was added to 10 ml medium. After overnight incubation cells were boosted with 10% DMSO for 30 min by adding 5 ml cold 30% DMSO in medium to each plate. Then the medium was removed and fresh medium added. Two days later the cells were passaged 1:4 and cultivated in medium containing 1.5 mg/ml G418 until colonies did appear (7–10 days). In the 'direct selection' experiments MNNG or HeCNU was given 1 day after reseeding in G418 medium. Colonies appearing 10 days after mutagen treatment were counted and the survival fraction determined in relation to the number of colonies that appeared on the non-mutagen-treated plates. In the 'reseeding selection' protocol neo$^+$ (G418^r) colonies were trypsinized and 10^6 cells seeded in medium without G418. Six hours later MNNG or HeCNU was added to the plates from a stock solution. Colonies were fixed, stained and counted 8–10 days later. Several human MGMT-expressing cell strains were established by picking colonies after direct selection with G418 and a single low dose of MNNG (2 μM) or HeCNU (60 μM). Cells were propagated routinely in medium containing G418 (1.5 mg/ml).

Northern blot analysis

Poly(A)$^+$ mRNA was extracted by lysis of cells in SDS buffer (0.5% SDS, 100 mM EDTA, 20 mM Tris–Cl, pH 7.4) and digestion with proteinase K for 1 h at 37°C. After extraction with chloroform/isoamyl alcohol (24:1) the solution was adjusted to 0.5 M NaCl and incubated overnight with oligo(dT)–cellulose (Type 7, Pharmacia; 100 mg/10^7 cells). After repeated washing with 0.1% SDS, 300 mM NaCl, 5 mM EDTA, 10 mM Tris–Cl, pH 7.5, the poly(A)$^+$ mRNA was eluted in distilled water and precipitated with ethanol. RNA was separated under denaturing conditions on glyoxal gels (52), transferred to a nylon membrane (Hybond N$^+$, Amersham) and hybridized with a random primed ^{32}P-labeled MGMT cDNA (according to manufacturer's protocol, Stratagene; 10^7 c.p.m./ml; 10^9 c p.m./μg DNA) in 7% SDS (Bio-Rad), 1 mM EDTA, 0.5 M phosphate buffer (pH 7.0). Filters were washed twice with 2 × SSC and once with 1 × SSC. Rehybridization of the filters was performed, after stripping, with a random primed ^{32}P-labeled glyceraldehyde-3-phosphodehydrogenase (GAPDH) probe (53).

Western blotting

About 10^8 exponentially growing cells were harvested by trypsinization, rinsed twice with PBS, resuspended in 1 ml buffer A (20 mM Tris, pH 8.6, 1 mM EDTA, 1 mM dithiotreitol, 10% glycerol) and homogenized with a French press (Pyrex, no. 7726). The suspension was centrifuged (10 min, 10 000 r.p.m.) and the supernatant saved. The pellet was resuspended in 1 ml 0.2 M NaCl in buffer A, incubated for 1 h on ice and centrifuged. The supernatants were combined and ammonium sulfate was added to 55% saturation. After 1 h on ice the precipitate was pelleted by centrifugation (15 min, 10 000 r.p.m.), resuspended in 0.5 ml buffer A and dialyzed against buffer A overnight. Samples with 50 μg of protein were loaded onto a 12% SDS–polyacrylamide gel and electrophoresed for 2 h at 10 mA. Proteins were electrophoretically transferred to a Nylon membrane (Immobilon-P, Millipore, Bedford, MA) and incubated with PBS, 5% non-fat dry milk and 0.1% Tween 20 and a monoclonal antibody specifically directed against the human MGMT protein (GAT 4.A1, ref. 54) for 1 h. Anti-mouse IgG conjugated to peroxidase (diluted 1/1500) was used as a secondary antibody (Bio-Rad). Peroxidase staining was performed according to manufacturers protocol.

Determination of MGMT activity

MGMT activity was determined in exponentially growing cells using two methods. (i) determination of transfer of ^{3}H activity from [^{3}H]O^6-methylguanine DNA template to protein essentially as previously described (55); (ii) analysis of conversion of O^6-methyl[^{3}H]guanine to [^{3}H]guanine in a synthetic O^6-methyl[^{3}H]guanine-containing DNA template by HPLC, after incubation of the template with cell extract as described (56). The number of cells used for extract preparation was determined with a hematocytometer. MGMT activity was expressed as molecules/cell.

Survival assays

Three hundred cells from exponentially growing cultures were seeded per 5 cm dish (two dishes for a given treatment and experiment) and treated 6 h later with the mutagen by adding the chemicals from a stock solution directly to the plates.

One hour after addition of the mutagens the medium was removed and fresh medium added. For UV irradiation the medium was removed, cells irradiated and fed with fresh medium. Colonies were fixed 7 days after seeding with methanol and stained with crystal violet. Survival frequency was expressed as number of colonies per treatment level/number of colonies in the control. The plating efficiency (control) was ~75%.

Mutation assays

Mutation induction at the hypoxanthine-guanine phosphoribosyltransferase locus (6-thioguanine resistance) was analyzed as previously described (57). Briefly, 2×10^5 cells were treated with the mutagens 1 day after seeding and replated twice during an expression period of 7 days. Cells (2×10^5) were seeded in medium containing 6-thioguanine (3 μg/ml) and, in parallel, 10^3 cells were seeded in non-selective medium for determination of plating efficiency. Colonies were fixed 7 days after seeding. Mutation frequency was expressed as number of 6-thioguanine resistant colonies/(number of cells seeded × plating efficiency).

Cytogenetic techniques

For assaying SCEs, 2×10^5 cells were seeded per 5 cm dish. Two days later mutagen was added, cells were incubated for 60 min, washed with PBS and fed with fresh medium containing 5-bromodeoxyuridine (10 μg/ml). Colcemid (50 ng/ml) was added 24 h later (after two generations) and after an additional 2 h cells were collected by trypsinization, hypotonized with 75 mM KCl and fixed with methanol/acetic acid (3:1, v/v). Chromosomes were differentially stained as previously described (57). At least 25 metaphases were evaluated per treatment level.

To determine the aberration frequency, exponentially growing cells were treated for 1 h with the mutagens. After a recovery period of 16 h cells were treated for 2 h with colcemid and harvested by trypsinization. Chromosomes were stained with 5% Giemsa (in PBS). At least 100 metaphases were evaluated per treatment level. Types of aberrations scored: g′, chromatid gaps; gg′, isochromatic gaps; b′, chromatid breaks; b″, isochromatid breaks; Q, quadriradials; T, triradials; dic, dicentrics; TT, all other types of reunited and non-reunited exchanges; d, intercalary deletions; dd, duplication deletions; mA, multiple aberrations (too many to score) per cell. Gaps were not included in the final evaluation.

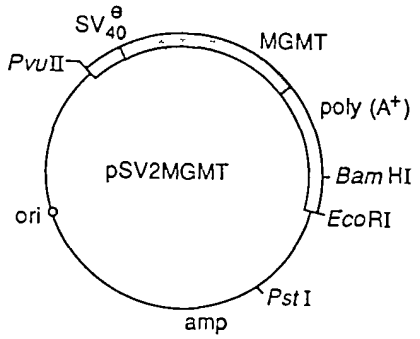

Fig. 1. Map of the expression plasmid *pSV2MGMT*. Not all of the available restriction sites are indicated. SV_{40}^e, early promoter of SV40.

Results

Survival of cells in mass culture transfected with pSV2ada or pSV2MGMT

To study whether and to what degree the human MGMT confers protection to the toxic effects of alkylating agents, CHO-9 cells were transfected with the MGMT cDNA cloned behind the SV40 promoter (plasmid *pSV2MGMT*, Figure 1). For control, transfections were performed with *pSV2neo* (and salmon sperm DNA) only. Two protocols were used for selection. In the 'direct mutagen selection' protocol (Table IA) cells were grown, 3 days upon transfection, in medium containing G418 only (control) and G418 plus MNNG or HeCNU. In the 'reseeding protocol' (Figure 1B) the $G418^r$ clones that were selected with G418 only were trypsinized and the cells reseeded in medium containing MNNG or HeCNU. As shown in Table I(A), 67−81% of the *pSV2MGMT* + *pSV2neo* transfectants survived the treatment with MNNG or HeCNU at doses that killed all (for HeCNU) or ~98% (for MNNG) of the cells transfected with *pSV2neo* only. To compare the ability of the human MGMT with the Ada protein in mediating killing protection, cells were co-transfected in parallel with the *ada* gene controlled by the SV40 promoter (*pSV2ada*). In all experiments performed, fewer survivors were obtained (30−58%) after *pSV2ada* transfection than after *pSV2MGMT* transfection.

Basically the same results have been obtained using the reseeding protocol. Co-transfection of *pSV2neo* with *pSV2MGMT* gave rise to a dramatic increase of survivors, after treating $G418^r$ cells with high doses of MNNG or HeCNU. Cells that had been transfected with *pSV2neo* + *pSV2ada* were also highly protected from killing by MNNG and HeCNU, but the level of protection was clearly lower than that for *pSV2MGMT* (Table IB).

Table I. Survival frequency of CHO-9 cells transfected with *pSV2neo*, *pSV2neo* + *pSV2MGMT* or *pSV2neo* + *pSV2ada* and treated with MNNG or HeCNU

(A) Plasmid	Average no. of neo^+ colonies/plate (control)	$G418^r$ colonies (% of control)			
		5 μM MNNG	6 μM MNNG	70 μM HeCNU	80 μM HeCNU
pSV2neo	279	2 4	1.7	0	0
pSV2neo + *pSV2MGMT*	287	66 6	70.2	81.4	72.6
pSV2neo + *pSV2ada*	327	58.4	49.8	36.5	29.8

(B) Plasmid	Frequency of survivors ($\times 10^{-5}$)	
	12.5 μM MNNG	90 μM HeCNU
pSV2neo	2.7	0
pSV2neo + *pSV2MGMT*	808	914
pSV2neo + *pSV2ada*	664	640

(A) Mutagen treatment during G418 selection. Values represent the percentage of G418-resistant colonies (as compared to the non-mutagen-treated control population). They are the mean of three independent experiments.

(B) Mutagen treatment after reseeding of G418 resistant colonies. Cells were seeded (10^5/plate) and treated 6 h later with the mutagens with the concentrations indicated (three plates per treatment level). Colonies were counted after 7 days. Values are given as survivors per 10^5 cells seeded. All data are the mean of three independent experiments.

Similar results have been obtained using Mex$^-$ HeLa M cells for transfection (data not shown). Because the MGMT and the *ada* gene were cloned into the same expression vector and were transfected under the same conditions (using the same amounts of *pSV2neo* + carrier DNA), we conclude that expression of the human gene is more efficient than the bacterial *ada* gene in conferring alkylation resistance in mammalian cells.

Generation of stable pSV2MGMT transfected clones

In order to study the protective effect of MGMT expression on the genotoxicity of various mutagens in more detail, cell clones were isolated after transfection with *pSV2neo* + *pSV2MGMT* (Table II). The cell strains generated express various amounts of MGMT ranging from 8670 to 209 260 molecules per cell (which are equivalent, in CHO-9 cells, to 45 and 1090 fmol/mg protein respectively). With the exception of strain TK40-AT2 which was MGMT deficient, all *pSV2MGMT* transfectants expressed MGMT-specific transcripts, the amount of which roughly paralleled their MGMT activity (Figure 2 and data not shown). That the MGMT was encoded by human cDNA in the transfectants was not only shown by Northern, but also by Western blot analysis. The transfectant strains used for these studies showed a distinct band corresponding to ~22 kDa, which is the mol. wt of the human MGMT protein (25,44). For the immunoblot a monoclonal antibody was used that reacts specifically with the human MGMT protein (54). There was no signal both in *pSV2neo*-transfected Mex$^-$ CHO cells and in Mex$^+$ rat H4IIE cells (harboring constitutively 138 000 molecules/cell; ref. 26). However, the antibodies reacted clearly with the MGMT protein purified from HeLa S3 cells (used as a positive control) (Figure 2). It should be noted that the Western blots do not allow quantification of MGMT because cell extracts were subjected to ammonium sulfate precipitation in order to enrich the MGMT protein.

The following cell strains have been used as controls for subsequent studies: non-transfected CHO-9 cells, cells transfected with *pSV2neo* only (CHO-9-neo-C5), and cells transfected with *pSV2neo* + *pSV2MGMT* and selected as described for MGMT transfectants, that possessed neither MGMT mRNA (not shown) nor MGMT activity (strain designated Tk40-AT2; see Table II).

Table II. Cell strains used to study the effect of MGMT expression on the genotoxicity of alkylating agents

Strain	Symbol	Transfected genes	MGMT activity (molecules/cell)
CHO-9	●	none	BD
CHO-9-neo-C5	■	*pSV2neo*	BD
CHO-9-ada-C4	*	*pSV2neo* + *pSV2ada*	345 000
Tk40-AT2	◑	*pSV2neo* + *pSV2MGMT*	BD
Tk40-AT4	○	*pSV2neo* + *pSV2MGMT*	58 650
Tk40-AT6	△	*pSV2neo* + *pSV2MGMT*	61 540
Tk40-AT8	□	*pSV2neo* + *pSV2MGMT*	41 820
Tk38-AT10/1-C1	▭	*pSV2neo* + *pSV2MGMT*	11 390
Tk38-AT10/1-C2	▽	*pSV2neo* + *pSV2MGMT*	14 620
Tk38-AT6/1-C2	◇	*pSV2neo* + *pSV2MGMT*	8 670
Tk47-AT17-C1	□	*pSV2neo* + *pSV2MGMT*	113 730
Tk47-AT17-C2	×	*pSV2neo* + *pSV2MGMT*	106 160
Tk47-AT17/C3	+	*pSV2neo* + *pSV2MGMT*	209 260

MGMT activity is given by the mean of three or four independent determinations. BD, below level of detection ($<10^3$ molecules/cell). The symbols given in the table for each strain are used throughout these studies (Figures 2–8).

To compare protection provided by MGMT with Ada in the expressors, in most experiments a strain generated upon transfection with *pSV2neo* + *pSV2ada* (CHO-9-ada-C4) was included. These cells express Ada, as shown by mRNA blot hybridization and immuno-blotting using an antibody directed against Ada (not shown). All strains have a stable phenotype; the methyltransferase activity remained constant over a period of months of cultivation.

Survival of MGMT transfectants upon mutagen exposure

Survival curves for various high (Tk47-AT17-C1, Tk47-AT17-C3) and low MGMT expression stains as well as an *ada* transfectant (CHO-9-ada-C4) are shown in Figures 3 and 4. It is obvious that expression of human MGMT in CHO cells renders them highly resistant to the toxic effects of HeCNU, MNNG and MNU.

For strain Tk47-AT17-C3, which has the highest MGMT activity, the increase in resistance to these agents was ~8-fold (as compared to CHO-9 or CHO-9-neo-C5 on the basis of D_{50} values). There was a slight increase of survival both of the MGMT and *ada* expressors after MMS and EMS treatment. No protection was observed for UV and, surprisingly, for ENU. Transfection of CHO-9 cells with the *neo* gene only and selection with G418 did not alter their sensitivity to all of the agents tested (Figure 4 and data not shown).

For MNNG and HeCNU, killing protection was analyzed in most of the transfectants listed in Table II that have low and high MGMT activity. This enabled us to study the level of protection afforded by different cellular amounts of MGMT. As shown in Figure 5, for strains expressing low and moderate MGMT activities (up to 60 000 molecules/cell) there was a nearly linear relationship between MGMT level and mutagen resistance. For the high MGMT expressors, however, there was no adequate increase of resistance with MGMT activity. Thus, it appears that

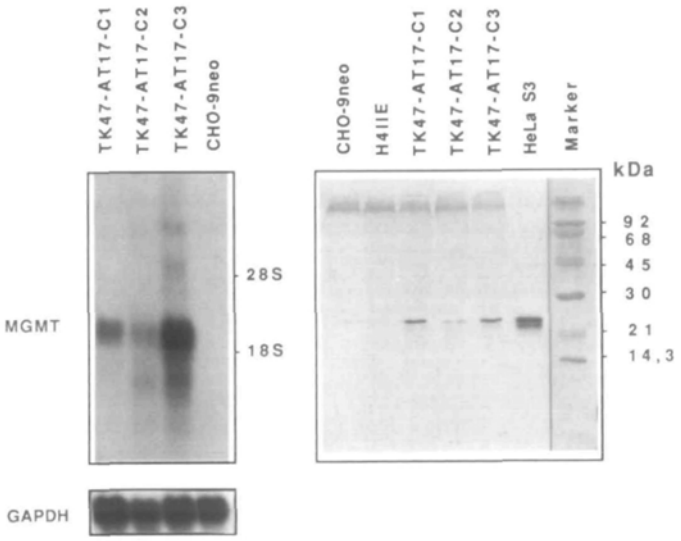

Fig. 2. Expression of human MGMT in CHO cells. (**Left**) Blot hybridization of mRNA of CHO cells transfected with *pSV2neo* only (CHO-9neo) or *pSV2neo* + *pSV2MGMT* (Tk47-AT17-C1, C2 and C3), with ^{32}P-labeled MGMT cDNA. The filter was rehybridized with GAPDH cDNA to demonstrate the presence of mRNA. (**Right**) Detection of human MGMT protein in CHO cells transfected with *pSV2MGMT* by immunoblotting. For controls, extracts of CHO-9neo (Mex$^-$), rat H4IIE (Mex$^+$) and human HeLa S3 (Mex$^+$) cells were subjected to electrophoresis on the same gel.

MGMT protects cells only to a certain level of toxicity of MNNG and HeCNU and that with high mutagen doses protection provided by MGMT becomes significantly less efficient.

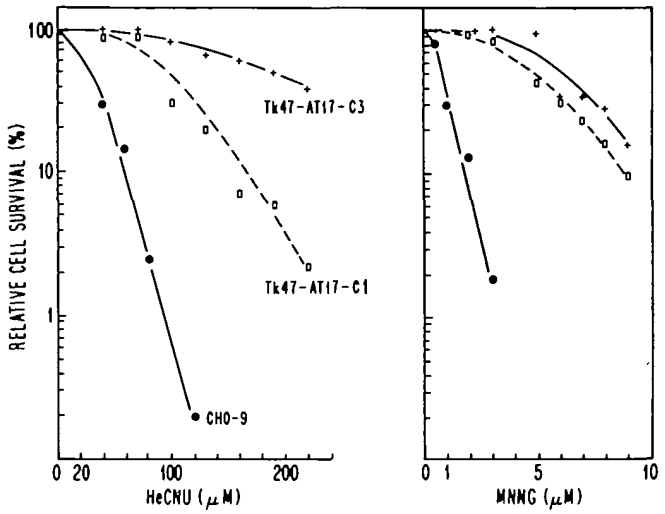

Fig. 3. Survival of CHO-9 and the MGMT transfectants Tk47-AT17-C1 and Tk47-AT17-C3 as a function of HeCNU and MNNG concentration.

MGMT protects from induction of gene mutations

Mutation induction at the HGPRT locus was analyzed in CHO-9-neo-C5 (control), the MGMT transfectants Tk40-AT4, Tk40-AT8, Tk38-AT6/1-C2, Tk38-AT10/1-C1, and, for comparison, CHO-9-ada-C4. As shown in Table III, in the transfectant strains expressing >40 000 MGMT molecules/cell (Tk40-AT4, Tk40-AT8, CHO-9-adaC4), mutation frequencies after MNNG treatment returned to the level of the non-mutagen-treated control (100% protection). Also for ENU, there was a clear reduction of induced mutations (by ~90% for ada-C4 and ~80% for strains AT4 and AT8). Obviously, MGMT (and the Ada protein) protects cells from the mutagenic effects of both methylating and ethylating agents.

Effects of MGMT expression on SCE and aberration induction

The induction of SCEs as a function of dose of various methylating and ethylating mutagens in CHO-9, CHO-9-neo-C5, CHO-9-ada-C4 and various MGMT transfectants is shown in Figure 6. The high MGMT expressors as well as the *ada* transfectant responded only very slightly with increase of SCE frequency to treatment with MNNG or MNU at doses that efficiently induced SCEs in the controls. The strain Tk38-AT6/1-C2, which expressed low MGMT activity, showed an intermediate sensitivity to MNNG. Contrary to MNNG and MNU, both the *ada*- and the MGMT-transfected cells were only

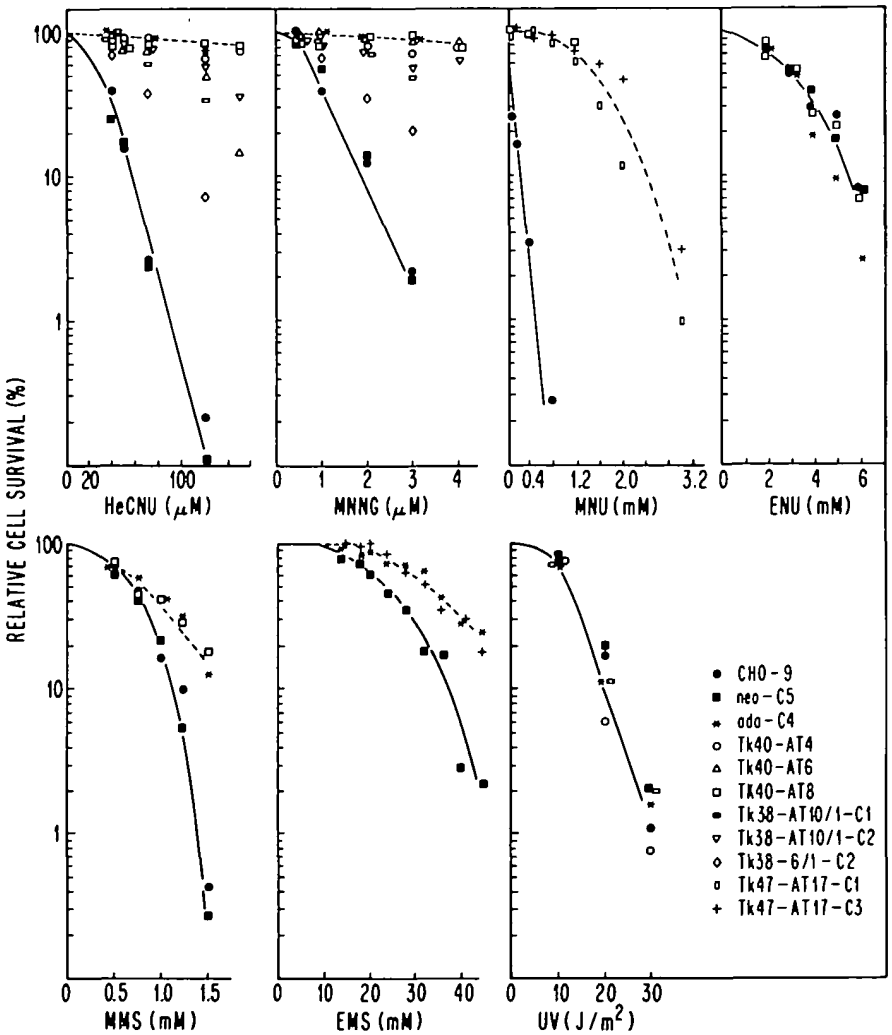

Fig. 4. Survival of CHO-9, CHO-9 transfected with *pSV2neo* (neo-C5), *pSV2neo* + *pSV2ada* (ada-C4) or *pSV2neo* + *pSV2MGMT* (Tk-strains) after exposure to various mutagens. For most treatments each point represents the mean of at least three independent experiments.

slightly protected from SCE induction by MMS and EMS. For ENU no reduction of SCE frequency was observed in both MGMT- and *ada*-expressing strains.

In Figure 7 (and the Appendix) aberration frequencies of contols and *ada* and MGMT transfectants treated with various methylating and ethylating agents are summarized. It is evident from the dose−response curves that chromosomal aberrations were less frequently induced by MNNG and MNU in methyltransferase-proficient cells. Protection pertained to all types of aberrations induced (not shown). There was a reduction in induced aberrations for MMS and EMS also, though not as strong as for MNNG. For ENU, again no protection was observed both in MGMT and *ada* expressors at the end-point aberration formation.

In order to analyze the correlation between the level of MGMT expression and the degree of protection, MNNG-induced mutation, SCE and aberration frequencies were plotted as a function of MGMT activity of the transfectants (Figure 8). It is obvious that with increasing amount of MGMT, both mutation and SCE frequency declined. With ≥40 000 MGMT molecules/cell the frequency of mutations was reduced nearly to control level (no mutation induction), and for SCEs the frequency reached a plateau at ~4 SCEs/cell. Apparently, in cells with MGMT levels <40 000 molecules the O^6-alkylguanine repair capacity is saturated, leaving O^6-methylguanine in DNA which may give rise to the generation of mutations and SCEs. Because the frequency of induced SCEs was not reduced below, on average, 4 SCEs/cell in high MGMT expressors (and cells expressing high amounts of the Ada protein), it is concluded that this residual level is due to lesions other than O^6-methylguanine. Based on this consideration, the contribution of O^6-methylguanine for SCE formation after MNNG or MNU treatment is estimated to range between 80 and 90%. For MMS and for the ethylating agents tested the relative contribution of O^6-alkylguanine to SCE formation is significantly lower (as deduced from dose−response curves shown in Figure 6).

The MGMT activity−effect curve for chromosomal aberrations showed, for high MGMT expressors, a plateau with an aberration frequency of ~40%, as compared to ~70% for Mex⁻ CHO cells (Figure 8). This plateau level is much higher than that observed for SCEs, indicating that O^6-methylguanine is less important for aberration than for SCE induction. Because the aberration frequency was not reduced to <40% even in high MGMT expressors, it is concluded that, in the case of MNNG, O^6-methylguanine contributes to ~40% to the induction of aberrations. For the other agents tested this value seems to be significantly lower, as deduced from the low level of protection (see dose−response curves and data shown in the Appendix).

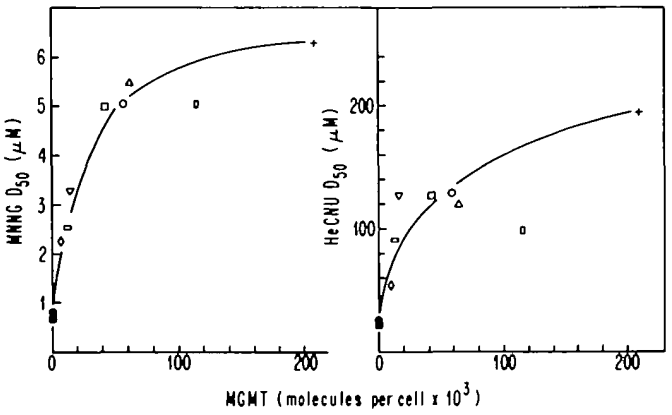

Fig. 5. Resistance of CHO Mex⁻ cells and MGMT transfectants to MNNG and HeCNU as a function of MGMT activity. The level of resistance is given by the dose reducing cell survival to 50% (D_{50}) Different symbols denote the cell strains used (see Table II).

Table III. Mutation frequencies of CHO-9-neo-C5 (Mex⁻ control), CHO-9-ada-C4 and *pSV2MGMT* transfectants (TK strains) not exposed and exposed to MNNG (1 μM) or ENU (2 mM)

Cell strain	Control	MNNG	ENU
CHO-9-neo-C5	5.8 ± 1.9 (3)	31.3 ± 15.2 (4)	171.3 ± 33 1 (3)
CHO-9-ada-C4	0.3 ± 0.3 (3)	0.6 ± 0.2 (3)	18.4 ± 13.7 (4)
Tk40-AT4	1.2 ± 0.7 (3)	1.8 ± 0.3 (3)	36.0 ± 30.5 (3)
Tk40-AT8	0.3 ± 0.2 (3)	0.6 ± 0.6 (3)	38 0 ± 18.5 (3)
Tk38-AT6/1-C2	0.2 ± 0.0 (3)	14.1 ± 9.1 (3)	ND
Tk38-AT10/1-C1	0.3 ± 0.1 (3)	5.4 ± 2.1 (3)	ND

Data are the mean of several independent experiments (number of experiments in brackets) and are given as mutants per 10^5 surviving cells ± SD. ND, not determined.

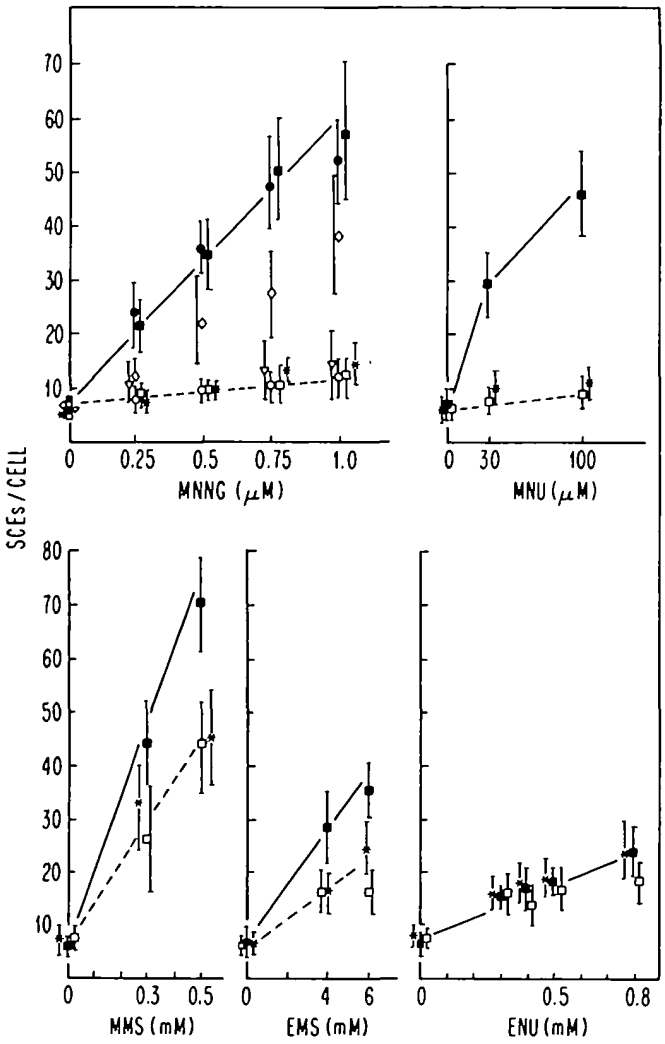

Fig. 6. SCE frequencies in CHO-9, CHO-9-neo-C5 (Mex⁻), CHO-9-ada-C4 and various MGMT expressing strains (for symbols see Table II) as a function of dose of MNNG, MNU, MMS, EMS and ENU. Data of six independent experiments are pooled. Each point represents the mean of at least 25 metaphases ± SD.

Discussion

Although O^6-methylguanine is one of the 'minor' lesions induced by alkylating agents [ranging from 0.3 to 9% of total alkyl adducts in DNA for MMS and ENU respectively (58)], several lines of evidence suggest that this lesion is critical in mutagenesis and malignant transformation (11). For other end-points such as cell killing, and induction of SCEs and aberrations, the contribution of O^6-methylguanine is less clear. Approaches to introduce selectively O^6-methylguanine in DNA failed (59–61). Therefore, the reverse approach, i.e. selective and variable removal of the adduct from DNA in an otherwise 'isogenic' set of cells, can provide compelling evidence on the role of the adduct in these phenomena. Based on this strategy, we have undertaken a series of experiments both with cells transfected and selected in mass culture, and with transgenic clones expressing widely different amounts of human MGMT. In the mass culture transfection and selection experiments clonal variations can be excluded; they easily permit a comparison of the ability of various plasmids to confer alkylation protection. The experiments reported here show that *pSV2MGMT* is very efficient in mediating alkylation protection. Up to 80% of the G418^r (neo$^+$) transfectants survived a MNNG or HeCNU treatment that was toxic for controls. In these experiments *pSV2MGMT* was more efficient that *pSV2ada* in conferring killing protection against MNNG and HeCNU. Because both plasmids are identical (both harbor the SV40 early promoter) except for the coding region, it is unlikely that this is due to differences in their expression. Probably MGMT is more efficient in repair than the Ada protein in mammalian cells, e.g. due to a better access of the human repair protein to DNA lesions. Other explanations are non-optimum conditions for Ada in mammalian cells, differences in nuclear localization or reduced stability of the protein.

The isolation and characterization of various clonal derivatives of MGMT overexpressing transfectants enabled us: (i) to analyze the correlation between the amount of MGMT and the level of protection for various end-points, and (ii) to compare the protective effect of MGMT towards different alkylating agents. Interestingly, only for low MGMT expressors (up to 80 000 molecules/cell) was there a roughly linear relation between MGMT activity and protection against cell killing. With activities >80 000 molecules/cell the level of resistance to MNNG and HeCNU was considerably lower than expected on the basis of a linear relationship. A reasonable interpretation for the non-linearity of the MGMT activity–response curve is that with increasing doses, lesions other than O^6-alkylguanine become the predominant toxic lesion. These are probably lesions that are not repaired or tolerated if induced at high levels due to saturation of the enzymatic system(s) involved. Non-linearity of the response may, in fact, be an explanation for some controversy about

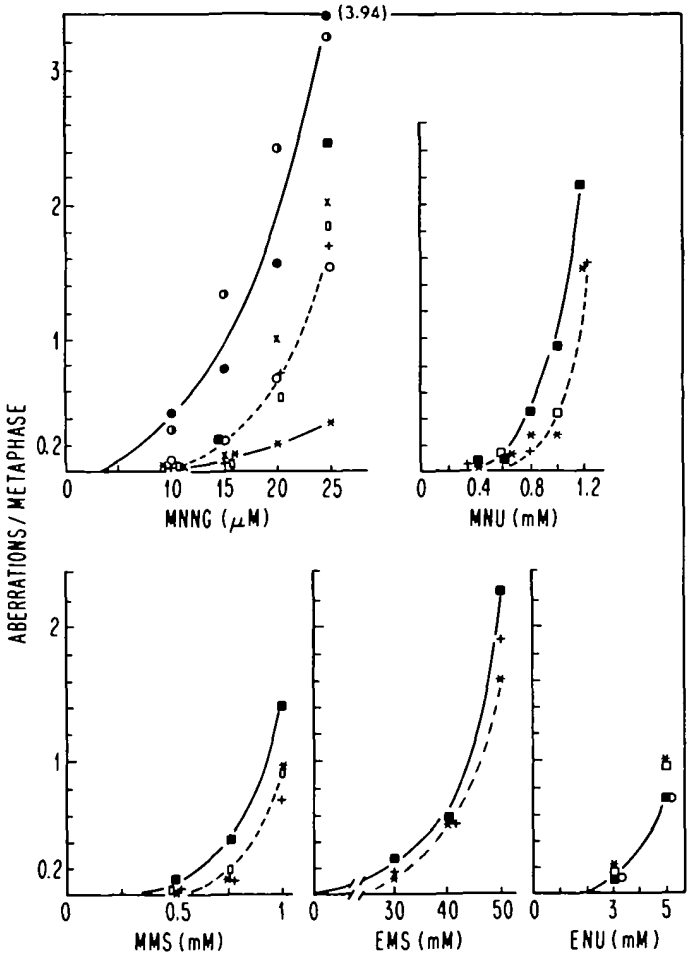

Fig. 7. Aberration frequencies in Mex$^-$ CHO cells (CHO-9 or CHO-9-neo-C5), CHO-9-ada-C4 and MGMT expressing strains (for symbols see Table II) as a function of dose of MNNG, MNU, MMS, EMS and ENU. The spontaneous aberration frequency for all strains was <0.02 aberrations/cell.

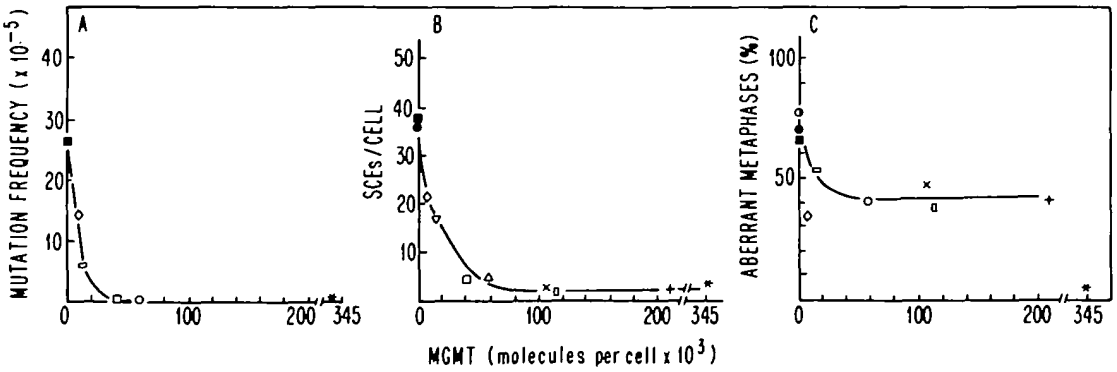

Fig. 8. MNNG-induced gene mutation (hypoxyanthine-guanine phosphoribosyltransferase deficiency). SCE and aberration frequency as a function of MGMT activity in Mex$^-$ CHO cells, ada-C4 and MGMT expressing strains (for symbols see Table II). Induced mutations: mutation frequency after treatment with 1 μM MNNG – mutation frequency of untreated control; induced SCEs: SCE frequency after treatment with 0.75 μM MNNG – SCE frequency of untreated control (see Appendix): induced aberrations: aberration frequency after treatment with 20 μM MNNG – aberration frequency of untreated control.

MGMT in killing protection and adaptation in mammalian cells. Killing protection against MNNG was repeatedly reported after complementation of Mex$^-$ cells with the *ada* gene (31,35) or total human DNA (62–65). No killing protection was observed when (i) depleting MGMT by feeding with O^6-methylguanine (66,67); (ii) expressing the *ada* gene in Mex$^+$ cells with high endogeneous MGMT activity (68); (iii) inducing MGMT transcription in rat hepatoma H4 cells (harbouring 130 000 molecules per cell) by X-rays or MNNG thus increasing MGMT activity by a factor of 2–3. In this case, cells were protected from the mutagenic but not toxic effect of a subsequent MNNG challenge dose (G.Fritz and B.Kaina, unpublished results and ref. 26).

It has been shown that in Mex$^+$ cells fewer SCEs were induced by MNNG than in Mex$^-$ cells, suggesting that O^6-methylguanine is an SCE-inducing lesion (69,70). Reduction of MNNG-induced SCEs was also observed in *ada*-transfected HeLa cells (32,33). On the other hand, analyzing Epstein–Barr virus-transformed lymphoblastoid cell lines (71), transformed NIH3T3 cells (72), or cells derived from different species (73), no strict inverse correlation has been found between MGMT activity and MNNG-induced SCEs. Our results clearly show that MGMT displays a strong protective effect on SCE induction by MNNG and MNU, and, to a lesser degree, to MMS and EMS. In case of MNNG and MNU, SCE frequency was only slightly enhanced above the control levels (SCE frequency in CHO-9- and *pSV2neo*-transfected cells) in high MGMT expressors (60–200 000 molecules/cell). This indicates that most (80–90%) of the SCEs generated after MNNG or MNU exposure were brought about by the presence of O^6-methylguanine in DNA; only a minor part is due to other (probably *N*-alkyl) adducts. For MMS, EMS and ENU, lesions other than O^6-alkylguanine seem to play the major part in SCE formation.

Previously we have shown that, for MNU and MNNG, SCEs were not significantly induced during the first post-treatment replication cycle, whereas for MMS, EMS and ENU, induction occurred both in the first and the second post-treatment cell cycle (49). From these data we concluded that secondary lesions are responsible for MNU- and MNNG-induced SCEs, whereas for the other agents both primary and secondary lesions are involved (49). O^6-Methylguanine appears to be the principal primary SCE-inducing lesion (for MNNG and MNU) that may give rise to a secondary lesion during replication. The nature of the primary damage, in addition to O^6-alkylguanine, that gives rise to SCE induction upon exposure to other alkylating agents like MMS, EMS and ENU remains to be established.

The protection afforded by MGMT against clastogenic effects of alkylating agents has not yet been studied in detail so far. Here we have shown that MGMT overexpressors are protected to some extent against the clastogenic effects of MNNG, MNU and, only very slightly, against MMS and EMS. Clastogenic protection was considerably lower than protection to cell killing, SCE and mutation induction. The MGMT activity–response curve for MNNG showed a maximal reduction by ~40% of the aberration yield of Mex$^-$ cells. This is considerably less than observed for SCEs, indicating that O^6-alkylguanine is involved to a significant lesser degree in aberration than SCE formation. Interestingly, a correlation was reported between clastogenicity and *N*-alkylation potency of simple alkylating agents (30). Thus, MMS, although inducing low relative yields of O^6-methylguanine, is a very potent clastogen. This and the low magnitude of clastogenic protection by MGMT supports the view that lesions other than O^6-methylguanine are most important for inducing aberrations. The contribution of O^6-alkylguanine to induction of aberrations seems to be dependent on the type of the clastogen. For MNNG a large fraction (~40%) of aberrations appears to be due to O^6-alkylguanine, for MMS and EMS its contribution is significantly lower.

An apparent exceptional response was obtained for ENU. Compared to other alkylating agents tested, ENU induces the highest relative amounts of O^6-alkylguanine (58). Surprisingly, no protection was afforded by MGMT to the toxic, SCE-inducing and clastogenic effects of this agent, though a clear reduction of ENU-induced mutations was observed. Lack of killing protection to ENU was also reported for Mex$^+$ rodent cells derived from a Mex$^-$ cell line (12). Lack of protection is very likely not an ethylation-specific matter. MGMT repairs, though with reduced efficiency, O^6-ethylguanine in DNA (45,74). Furthermore, protective effects of MGMT have been observed against EMS. ENU has a high ability to alkylate oxygen atoms in DNA and induces unusually high amounts of phosphotriesters (56% as compared to 12% for EMS; refs 3,58). It is supposed that, for ENU, this and/or other lesions (other than O^6-ethylguanine) predominate in giving rise to cell killing, SCE and aberration formation.

First indications of O^6-methylguanine as a toxic and SCE-inducing lesion were derived by comparing Mex$^+$ and Mex$^-$ human cell lines (69). These cells were, however, not isogenic and differed in several respects. Therefore the involvement of other alterations attributed to the Mex$^+$ phenotype (75) cannot be excluded in alkylation resistance of these cells. For example, the Mex$^+$ transfectant used in a previous study (76) was obtained after extensive exposure to 2-chloroethyl-*N*-nitrosourea, which could induce a number of genetic and epigenetic changes in the cell line. Indeed it has now been shown that the MGMT activity in the CHO Mex$^+$ line was encoded by the endogenous gene (77). By comparing a set of MGMT cDNA transfectants expressing widely different amounts of repair protein, compelling evidence has been provided that MGMT plays a key role in protection of cells from genotoxic effects of various alkylating agents. The results presented here confirmed and extend a recent report in that alkylating agents produce several classes of lethal lesions, one of which is O^6-alkylguanine (65). For MMS, EMS and ENU, lesion(s) other than O^6-alkylguanine are primarily involved in induction of cell death. For induction of gene mutations, however, O^6-alkylguanine seems to be the major adduct for all monofunctional alkylating agents.

The contribution of O^6-alkylguanine to diverse biological endpoints has been assessed on the basis that MGMT is responsible for the removal of this lesion. Recently it has been reported that the human MGMT, like its *E.coli* counterparts Ada and Ogt, can also demethylate O^4-methylthymine *in vitro* (78,79). Thus it could be argued that O^4-methylthymine induced by MNNG and related agents may (also) be an important genotoxic lesion. However, repair of O^4-methylthymine by the human MGMT *in vitro* is very inefficient compared to that of O^6-ethylguanine (79). Furthermore, *in vitro* repair of O^4-ethylthymine was not detected with an excess of human MGMT (A.Wani, S.Shiota and S.Mitra, unpublished). The low ability of MGMT to repair O^4-methylthymine seems also to be true under *in vivo* conditions since O^4-methylthymine has a much longer half-life than O^6-methylguanine in DNA (for review see ref. 80). Thus it is unlikely that O^4-methylthymine is a main toxic lesion and responsible for all of the genotoxic effects that were affected by MGMT expression.

MGMT is not the only function that is responsible for avoiding genotoxicity of alkylating agents. For instance, the mutant 27-1, a derivative of CHO-9, is hypersensitive to MNNG and MNU

(81,82). Both strains lack MGMT and display a 'normal' pattern of excision of main DNA alkylation products (83). Both 27-1 and CHO-9 have been complemented by transfection with human DNA, giving rise to MNNG^r/Mex$^-$ clones, indicating that tolerance mechanisms have been evoked in these transfectants (50,82). Similar conclusions have been derived from MNNG^r/Mex$^-$ cells obtained by mutagen selection only (84,85). Furthermore, expression of the human metallothioneine IIA gene in CHO cells that lack metallothioneine activity rendered them MNNG/MNU resistant (55). Apparently mammalian cells have evolved a cascade of mechanisms contributing to removal and tolerance of DNA lesions, thus avoiding genotoxic effects of alkylating agents. MGMT seems to play a major part in protection from mutagenic, carcinogenic and killing effects of various alkylating compounds.

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References

1. Peto,R., Gray,R., Brantom,P. and Grosso,P. (1985) *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer*, IARC Scientific Publication no. 57. IARC, Lyon, pp. 627–655.
2. Singer,B. (1975) The chemical effects of nucleic acid alkylation and their relation to mutagenesis and carcinogenesis. *Progr. Nucleic Acid Res. Mol. Biol.*, **15**, 219–284.
3. Singer,B. and Grunberger,D (1983) *Molecular Biology of Mutagens and Carcinogens*. Plenum Press, New York.
4. Loveless,A. (1969) Possible relevance of O^6-alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature*, **223**, 206–207.
5. Abbott,P.J. and Saffhill,R. (1979) DNA synthesis with methylated poly(dC-dG) templates. Evidence for a competitive nature to miscoding by O^6-methylguanine. *Biochim. Biophys. Acta*, **562**, 51–61.
6. Eadie,J S., Conrad,M., Toorchen,D and Topal,M.D. (1984) Mechanism of mutagenesis by O^6-methylguanine. *Nature*, **308**, 201–203.
7. Saffhill,R. (1985) *In vitro* miscoding of alkylthymines with DNA and RNA polymerases. *Chem.-Biol Interactions*, **13**, 121–130
8. Goth,R. and Rajewski,M.F. (1974) Persistance of O^6-ethylguanine in rat-brain DNA: correlation with nervous system specific carcinogenesis by ethylnitrosourea. *Proc. Natl. Acad. Sci. USA*, **71**, 639–643.
9. Kleihues,P. and Margison,G.P. (1974) Carcinogenicity of *N*-methyl-*N*-nitrosourea: possible role of excision of O^6-methylguanine from DNA. *J. Natl. Cancer Inst.*, **53**, 1839–1841.
10. De Kok,A.J., van Zeeland,A.A., Simons,J.W.I.M. and den Engelse,L. (1985) Genetic and molecular mechanisms of the *in vitro* transformation of Syrian hamster embryo cells by the carcinogen *N*-ethyl-*N*-nitrosourea. II. Correlation of morphological transformation, enhanced fibrinolytic activity, gene mutations, chromosomal alterations and lethality to specific carcinogen-induced DNA lesions. *Carcinogenesis*, **6**, 1571–1576.
11. Saffhill,R., Margison,G.P. and O'Connor,P.J. (1985) Mechanisms of carcinogenesis induced by alkylating agents. *Biochim. Biophys. Acta*, **823**, 111–145.
12. Thomale,J., Huh,N.H., Nehls,P., Eberle,G. and Rajewski,M.F. (1990) Repair of O^6-ethylguanine in DNA protects rat 208F cells from tumorigenic conversion by *N*-ethyl-*N*-nitrosourea. *Proc. Natl. Acad. Sci. USA*, **87**, 9883–9887.
13. Foote,R S., Mitra,S. and Pal,B.C. (1980) Demethylation of O^6-methylguanine in a synthetic DNA polymer by an inducible activity in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **97**, 654–659.
14. Demple,B., Jacobsson,A., Olsson,M., Robins,P. and Lindahl,R. (1982) Repair of alkylated DNA in *Escherichia coli*: physical properties of O^6-methylguanine-DNA methyltransferase. *J. Biol. Chem.*, **257**, 13776–13780.
15. Yarosh,D.B. (1985) The role of O^6-methylguanine-DNA methyltransferase in cell survival, mutagenesis and carcinogenesis. *Mutat. Res.*, **145**, 1–16.
16. Lindahl,T., Sedgwick,B., Sekiguchi,M. and Nakabeppu,Y. (1988) Regulation and expression of the adaptive response to alkylating agents. *Ann. Rev. Biochem.*, **57**, 133–157.
17. Demple,B. and Karran,P. (1983) Death of an enzyme: suicide repair of DNA. *Trends Biol. Sci.*, **8**, 137–139.
18. Grafstrom,R., Pegg,A.E., Trump,B.F. and Harris,C.C. (1984) O^6-Alkylguanine-DNA alkyltransferase activity in normal human tissues and cells. *Cancer Res.*, **44**, 2855–2857.
19. Gerson,S.L., Trey,J.E., Miller,K. and Berger,N.A. (1986) Comparison of O^6-alkylguanine-DNA alkyltransferase activity based on cellular DNA content in human, rat and mouse tissue. *Carcinogenesis*, **7**, 745–749.
20. Belinski,S.A., Dolan,M.E., White,C.W., Maronpot,R.R., Pegg,A.E. and Anderson,M.E. (1988) Cell specific differences in O^6-methylguanine-DNA methyltransferase activity and removal of O^6-methylguanine in rat pulmonary cells. *Carcinogenesis*, **9**, 2053–2058.
21. Washington,W.J., Foote,R.S., Dunn,W.C., Generoso,W.M. and Mitra,S. (1989) Age-dependent modulation of tissue-specific repair activity for 3-methyladenine and O^6-methylguanine in DNA in inbred mice. *Mech. Ageing Dev.*, **28**, 43–52.
22. Sklar,R.M. and Strauss,B.S. (1981) Removal of O^6-methylguanine from DNA of normal and Xeroderma pigmentosum-derived lymphoblastoid lines. *Nature*, **289**, 417–420.
23. Yarosh,D.B., Foote,R.S., Mitra,S. and Day,R.S.,III (1983) Repair of O^6-methylguanine in DNA by demethylation is lacking in Mer$^-$ human tumor strains. *Carcinogenesis*, **4**, 199–205.
24. Yarosh,D.B., Rice,M., Day,R.S., Foote,R S. and Mitra,S. (1984) O^6-Methylguanine-DNA methyltransferase in human cells. *Mutat. Res.*, **131**, 27–36.
25. Harris,A.L., Karran,P. and Lindahl,T. (1983) O^6-Methylguanine-DNA methyltransferase of human lymphoid cells: structural and kinetic properties and absence in repair deficient cells. *Cancer Res.*, **43**, 3247–3252.
26. Fritz,G., Tano,K., Mitra,S. and Kaina,B. (1991) Inducibility of the DNA repair gene encoding O^6-methylguanine–DNA methyltransferase in mammalian cells by DNA-damaging treatments. *Mol. Cell. Biol.*, **11**, in press.
27. Larson,K., Sahm,J., Shenkar,R. and Strauss,B. (1985) Methylation-induced blocks to *in vitro* DNA replication. *Mutat. Res.*, **150**, 77–84.
28. Karran,P., Hjilmgreen,T. and Lindahl,T. (1982) Induction of a DNA glycolase for *N*-methylation purines is part of the adaptive response to alkylating agents. *Nature*, **296**, 770–773.
29. Morris,S.M., Heflich,R.H., Beranek,D.T. and Kodell,R.L. (1982) Alkylation-induced sister-chromatid exchanges correlate with reduced cell survival, not mutations. *Mutat. Res.*, **105**, 163–168.
30. Vogel,E. and Natarajan,A.T. (1979) The relation between reaction kinetics and mutagenic action of mono-functional alkylating agents in higher eukaryotic systems. I. Recessive lethal mutations and translocations in *Drosophila*. *Mutat. Res.*, **62**, 51–100.
31. Kataoka,H., Hall,J. and Karran,P. (1986) Complementation of sensitivity to alkylating agents in *Escherichia coli* and Chinese hamster ovary cells by expression of a cloned bacterial DNA repair gene. *EMBO J.*, **5**, 3195–3200.
32 Samson,L., Derfler,B. and Waldstein,E.A. (1986) Suppression of human DNA alkylating-repair defects by *Escherichia coli* DNA-repair genes. *Proc. Natl. Acad. Sci. USA*, **83**, 5607–5610.
33. Ishizaki,K., Tsujimura,R., Yawata,H., Fujio,C., Nakabeppu,Y., Sekiguchi,M. and Ikenaga,M. (1986) Transfer of the *E.coli* O^6-methylguanine methyltransferase gene into repair-deficient human cells and restoration of cellular resistance to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Mutat. Res.*, **166**, 135–141.
34. Brennand,J. and Margison,G.P. (1986) Reduction of the toxicity and mutagenicity of alkylating agents in mammalian cells harbouring the *Escherichia coli* alkyltransferase gene. *Proc. Natl. Acad. Sci. USA*, **83**, 6292–6296.
35. White,G.R.M., Ockey,C.H., Brennand,J. and Margison,G.P. (1986) Chinese hamster cells harbouring the *Escherichia coli* O^6-alkylguanine alkyltransferase gene are less susceptible to sister chromatid exchange induction and chromosome damage by methylating agents. *Carcinogenesis*, **7**, 2077–2080.
36. Kaina,B., Fritz,G. and Coquerelle,T. (1981) Identification of human genes involved in repair and tolerance of DNA damage. *Radiat. Environ. Biophys.*, **30**, 1–19.
37. McCarthy,T.V., Karran,P. and Lindahl,T. (1984) Inducible repair of *O*-alkylated DNA pyrimidines in *Escherichia coli*. *EMBO J.*, **3**, 545–550.
38. McCarthy,T.V. and Lindahl,T. (1985) Methylphosphotriesters in alkylated DNA are repaired by the Ada regulatory protein of *E.coli*. *Nucleic Acids Res.*, **13**, 2683–2698.
39. Teo,I., Sedgwick,B., Kilpatrick,M.W., McCarthy,T.V and Lindahl,T. (1986) The intracellular signal for induction of resistance to alkylating agents in *E.coli*. *Cell*, **45**, 315–324

40. Brennand,J. and Margison,G.P. (1986) Expression in mammalian cells of a truncated *Escherichia coli* gene coding for O^6-alkylguanine alkyltransferase reduces the toxic effects of alkylating agents. *Carcinogenesis*, **7**, 2081–2084.
41. Ishizaki,K., Tsujimura,T., Fujio,C., Yangpei,Z., Yawata,H., Nakabeppu,Y., Sekiguchi,M. and Ikenaga,M. (1987) Expression of the truncated *E.coli* O^6-methylguanine methyltransferase gene in repair-deficient human cells and restoration of cellular resistance to alkylating agents. *Mutat. Res.*, **184**, 121–128.
42. Hall,J., Kataoka,H., Stephenson,C. and Karran,P. (1988) The contribution of O^6-methylguanine and methylphosphotriesters to the cytotoxicity of alkylating agents in mammalian cells. *Carcinogenesis*, **9**, 1587–1593.
43. Pegg,A.E,. Dolan,M.E., Scicchitano,D. and Morimoto,K. (1985) Studies of the repair of O^6-alkylguanine and O^4-alkylthymine in DNA by alkyltransferases from mammalian cells and bacteria. *Environ. Health Perspect.*, **62**, 109–114.
44. Bhattacharyya,D , Foote,R.S., Boulden,A.M and Mitra,S. (1990) Physicochemical studies of human O^6-methylguanine-DNA methyltransferase. *Eur. J. Biochem.*, **193**, 337–343.
45. Pegg,A.E, Wiest,L., Foote,R.S., Mitra,S. and Perry,W. (1988) Purification and properties of O^6-methylguanine-DNA transmethylase from rat liver. *J. Biol. Chem.*, **258**, 2327–2333.
46. Tano,K., Shiota,S., Collier,J., Foote,R.S. and Mitra,S. (1990) Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O^6-alkylguanine. *Proc. Natl. Acad. Sci. USA*, **87**, 686–690.
47. Rydberg,B., Spurr,N. and Karran,P. (1990) cDNA cloning and chromosomal assignment of the human O^6-methylguanine-DNA methyltransferase. cDNA expression in *Escherichia coli* and gene expression in human cells *J. Biol. Chem.*, **265**, 9563–9569.
48. Hayakawa,H., Koike,G. and Sekiguchi,M. (1990) Expression and cloning of complementary DNA for a human enzyme that repairs O^6-methylguanine in DNA. *J. Mol. Biol.*, **213**, 739–747.
49. Kaina,B. and Aurich,O. (1985) Dependency of the yield of sister-chromatid exchanges induced by alkylating agents on fixation time. Possible involvement of secondary lesions in sister-chromatid exchange induction *Mutat. Res.*, **149**, 451–461.
50. Kaina,B., van Zeeland,A.A., Backendorf,C., Thielmann,H.W. and van de Putte,P. (1987) Transfer of human genes conferring resistance to methylating mutagens, but not to UV irradiation and cross-linking agents, into Chinese hamster ovary cells. *Mol. Cell. Biol.*, **7**, 2024–2030.
51. Nakebeppu,Y., Kondo,H., Kawebata,S., Imagawa,S. and Sekiguchi,M. (1985) Purification and structure of the intact ada regulatory protein of *Escherichia coli* K12, O^6-methylguanine-DNA methyltransferase. *J. Biol. Chem.*, **260**, 2781–2788.
52. McMaster,G.K. and Carmichael,G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA*, **74**, 4835–4838
53. Fort,P., Marty,L., Piechaczyk,M., Elsabrouty,S., Dani,C., Jeanteur,P. and Blanchard,J.M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate dehydrogenase multigenic family. *Nucleic Acids Res.*, **13**, 1431–1442.
54. Brent,T.P., von Wronski,M., Pegram,C.N. and Bigner,D.D. (1990) Immunoaffinity purification of human O^6-alkylguanine-DNA alkyltransferase using newly developed monoclonal antibodies. *Cancer Res.*, **50**, 58–61
55. Kaina,B., Lohrer,H., Karin,M. and Herrlich,P. (1990) Overexpressed human metallothionein IIA gene protects Chinese hamster ovary cells from killing by alkylating agents. *Proc. Natl. Acad. Sci. USA*, **87**, 2710–2714.
56. Foote,R.S. and Mitra,S. (1984) Lack of induction of O^6-methylguanine-DNA methyltransferase in mammalian cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Carcinogenesis*, **5**, 277–281.
57. Kaina,B. (1983) Studies on adaptation of V79 Chinese hamster cells to low doses of methylating agents. *Carcinogenesis*, **4**, 1437–1443.
58. Beranek,D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res.*, **231**, 11–30.
59. Ball,J.C., McCormick,J.J. and Maher,V.H. (1983) Biological effects of incorporation of O^6-methyldeoxyguanosine into Chinese hamster V79 cells. *Mutat. Res.*, **110**, 423–433.
60. Kaina,B. and Aurich,O. (1984) Mutagenic and genotoxic effects of O^6-methylguanine in Chinese hamster cells: a revision. *Mutat. Res* , **139**, 149–154.
61. Foote,R.S., Dunn,W.C., Stankowski,L.F., Hsie,A.W. and Mitra,S. (1988) Mutagenic potential of modified DNA precursors *in vivo*: fate of O^6-methyl-deoxyguanosine triphosphate in Chinese hamster ovary cells. *Ind. J. Biochem. Biophys.*, **25**, 472–477.
62. Yarosh,D.B., Barnes,D. and Erickson,L.C. (1986) Transfection of DNA from a chloroethylnitrosourea-resistant tumor cell line (MER^+) to a sensitive tumor cell line (MER^-) results in a tumor cell line resistant to MNNG and CNU that has increased O^6-methylguanine-DNA methyltransferase levels and reduced levels of DNA interstrand crosslinking. *Carcinogenesis*, **7**, 1603–1606.
63. Barrows,L.R., Borchers,A.H. and Paxton,M B. (1987) Transfectant CHO cells expressing O^6-alkylguanine-DNA-alkyltransferase display increased resistance to DNA damage other than O^6-guanine alkylation. *Carcinogenesis*, **8**, 1853–1859.
64. Dolan,M.E , Norbeck,L., Clyd,C., Hora,N.K., Erickson,L.C. and Pegg,A.E. (1989) Expression of mammalian O^6-alkylguanine-DNA alkyltransferase in a cell line sensitive to alkylating agents. *Carcinogenesis*, **10**, 1613–1619.
65. Dunn,W.C., Tano,K., Horesovsky,G.J., Preston,R.J. and Mitra,S. (1991) The role of O^6-alkylguanine in cell killing and mutagenesis in Chinese hamster ovary cells. *Carcinogenesis*, **12**, 83–89.
66. Karran,P. and Williams,S.A. (1985) The cytotoxic and mutagenic effects of alkylating agents on human lymphoid cells are caused by different DNA lesions. *Carcinogenesis*, **6**, 789–792.
67. Domoradzki,J . Pegg,A.E., Dolan,M.E , Maher,V H. and McCormick,J J. (1985) Depletion of O^6-methylguanine-DNA methyltransferase in human fibroblasts increases the mutagenic response to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Carcinogenesis*, **6**, 1823–1826.
68. Dumenco,L.L., Warman,B., Hatzoglon,M., Lim,I.K., Abbond,S.L and Gerson,S L. (1989) Increase in nitrosourea resistance in mammalian cells by retrovirally mediated gene transfer of bacterial O^6-alkylguanine-DNA alkyltransferase *Cancer Res.*, **49**, 6044–6051.
69. Day,R.S.,III, Ziolkowski,C.H.J., Scudiero,D.A., Mayer,S.A., Lubinieckı,A.S., Girardı,A.J., Galloway,S.M. and Bynum,G.D. (1980) Defective repair of alkylated DNA by human tumor and SV40-transformed human cell strains. *Nature*, **288**, 724–727.
70. Samson,L. and Linn,S. (1987) DNA alkylation repair and the induction of cell death and sister chromatid exchange in human cells. *Carcinogenesis*, **8**, 227–230.
71. Schwartz,J.L., Turkula,T , Sagher,D. and Strauss,B. (1989) The relationship between O^6-alkylguanine alkyltransferase activity and sensitivity to alkylation-induced sister chromatid exchanges in human lymphoblastoid cell lines. *Carcinogenesis*, **10**, 681–685
72. Mitani,H , Ito,K., Fujino,M. and Takebe,H. (1987) Difference in O^6-methylguanine methyltransferase activity among transformed NIH3T3 cell clones. *Mutat. Res.*, **191**, 201–205.
73. Ikenaga,M., Tsujimura,T., Cheng,H.R., Fujio,C., Zhang,Y.P., Ishizaki,K., Kataoka,H. and Shima,A. (1987) Comparative analysis of O^6-methylguanine methyltransferase activity and cellular sensitivity of alkylating agents in cell strains derived from a variety of animal species. *Mutat. Res.*, **184**, 161–168.
74. Sedgwick,B. and Lindahl,T (1982) A common mechanism for repair of O^6-methylguanine and O^6-ethylguanine in DNA. *J. Mol. Biol.*, **154**, 169–175.
75. Karran,P. and Lindahl,R. (1985) Cellular defence mechanisms against alkylating agents *Cancer Surv.*, **4**, 583–599.
76. Bignami,M., Terlizzese,M., Zijno,A., Calcagnile,A., Frosina,G., Abbondandolo,A. and Dogliotti,E. (1987) Cytotoxicity, mutations and SCEs induced by methylating agents are reduced in CHO cells expressing an active mammalian O^6-methylguanine-DNA methyltransferase gene. *Carcinogenesis*, **8**, 1417–1421.
77. Tano,K., Shiota,S., Remack,J.S., Brent,T.P., Bigner,D.D. and Mitra,S. (1991) The origin of O^6-methylguanine-DNA methyltransferase in Chinese hamster ovary cells transfected with human DNA. *Mutat. Res.*, in press
78. Koike,G., Maki,H., Takeya,H., Hayakawa,H. and Sekiguchi,M. (1990) Purification, structure and biochemical properties of human O^6-methylguanine-DNA methyltransferase. *J. Biol. Chem.*, **265**, 14754–14762.
79. Sassanfar,M., Dosanjh,M.K., Essignmann,J.M. and Samson,L. (1991) Relative efficiencies of the bacterial, yeast, and human DNA methyltransferases for the repair of O^6-methylguanine and O^4-methylthymine. *J. Biol. Chem.*, **266**, 2767–2771.
80. Brent,T.P., Dolan,M.E., Frankel-Conrat,H., Hall,J., Karran,P., Laval,F., Margison,G.P., Montesano,R., Pegg,A.E., Potter,P.M., Singer,B., Swenberg,J.A. and Yarosh,D.B. (1988) Repair of *O*-alkylpyrimidines in mammalian cells: a present consensus. *Proc. Natl. Acad. Sci. USA*, **85**, 1759–1762.
81. Zdzienicka,M.Z. and Simons,J.W.I.M. (1986) Analysis of repair processes by the determination of the induction of cell killing and mutations in two repair-deficient Chinese hamster ovary cell lines. *Mutat. Res.*, **166**, 59–69.
82. Kaina,B. (1987) Correction of alkylation hypersensitivity of CHO-27-1 cells by transfection with human DNA. *Carcinogenesis*, **8**, 1935–1938.
83. Kaina,B., van Zeeland,A.A., de Groot,A. and Natarajan,A.T. (1990) DNA repair and chromosomal stability in the alkylating agent-hypersensitive Chinese hamster cell line 27-1. *Mutat. Res.*, **243**, 219–224.

84. Goth-Goldstein,R. (1987) MNNG-induced partial reversion of Mer⁻ cells *Carcinogenesis*, **8**, 1449–1453.
85. Green,M H.L., Lowe,J.E , Petit-Frère,C., Karran,P., Hall,J. and Kataoka,H. (1989) Properties of *N*-methyl-*N*-nitrosourea-resistant, Mex⁻ derivatives of an SV40 immortalized human fibroblast cell line. *Carcinogenesis*, **10**, 893–898.

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Appendix

Table AI. SCE frequencies in CHO cells not treated and treated with 0.75 μM MNNG (60 min)

Strain	Control	MNNG	Strain	Control	MNNG
CHO-9	5.9 ± 1.7	42.2 ± 9 3	TK38-AT10/1-C1	ND	ND
CHO-9-neo-C5	6.6 ± 2.2	45.6 ± 8.3	TK38-AT10/1-C2	6.3 ± 1.8	13.3 ± 8.7
CHO-9-ada-C4	6 2 ± 2.2	13 7 ± 2.5	TK38-AT6/1-C2	5.8 ± 1.6	27.3 ± 8.7
TK40-AT2	6.4 ± 2.8	45.1 ± 6.3	TK47-AT17-C1	5.8 ± 1.8	7.2 ± 2 7
TK40-AT4	5.5 ± 1.6	11.0 ± 3.6	TK47-AT17-C2	5.6 ± 2.2	8.4 ± 2.3
TK40-AT6	ND	ND	Tk47-AT17-C3	9.2 ± 2.2	11.7 ± 3.3
TK40-AT8	6.2 ± 2 0	10.5 ± 3.2			

For all strains data of one representative experiment are shown (25 metaphases ± SD). ND, not determined.

Table AII. Aberration frequencies of CHO-9, CHO-9-neo-C5 (Mex⁻), CHO-9-ada-C4 and various pSV2MGMT transfectants after exposure to MNNG, ENU, MNU, EMS and MMS

Mutagen	Treatment	CHO-9	CHO-9-neo-C5	ada-C4	AT4	AT8	AT17-C3	AT17-C1	AT17-C2	AT2
MNNG	control	0 (0)	1 (0.1)	2 (0.03)	0 (0)	0 (0)	1 (0.01)	0 (0)	0 (0)	2 (0.02)
	10 μM	30 (0.45)	10 (0.11)	2 (0.05)	7 (0.09)	4 (0.04)	2 (0.02)	4 (0.05)	3 (0.03)	21 (0.33)
	15 μM	41 (0.77)	17 (0.22)	6 (0.11)	19 (0.24)	23 (0.29)	7 (0.07)	5 (0.06)	12 (0.13)	54 (1.34)
	20 μM	70 (1.55)	65 (1 45)	4 (0.29)	41 (0.70)	28 (0.50)	40 (0.73)	38 (0.57)	47 (0.99)	78 (2.42)
	25 μM	96 (3.94)	90 (2.46)	17 (0.37)	72 (1.55)	42 (0.99)	74 (1.68)	74 (1.84)	70 (2.14)	92 (3.24)
ENU	control		0 (0)	0 (0)	1 (0 01)	0 (0)				
	3 mM		9 (0.12)	16 (0.19)	9 (0.10)	11 (0.15)				
	5 mM		52 (0.71)	60 (1.08)	45 (0 70)	47 (0.96)				
MNU	control		0 (0)	1 (0 01)		1 (0.1)	0 (0)			
	0.4 mM		7 (0 07)	2 (0.02)			4 (0.04)			
	0.6 mM		7 (0.08)	10 (0.12)		9 (0.11)				
	0.8 mM		40 (0.44)	7 (0 28)		24 (0.44)				
	1 mM		28 (0.94)	16 (0.28)						
	1.2 mM		80 (2.14)	56 (1 52)			54 (1.54)			
EMS	control		0 (0)	0 (0)			1 (0.01)			
	30 mM		26 (0.28)	8 (0.10)			15 (0.16)			
	40 mM		40 (0.58)	19 (0.52)			32 (0.52)			
	50 mM		84 (2.26)	66 (1.60)			70 (1.90)			
MMS	control		0 (0)	0 (0)			1 (0.01)	0 (0)	0 (0)	
	0.5 mM		10 (0.11)	4 (0.04)			2 (0.02)	8 (0.09)	3 (0.03)	
	0.75 mM		26 (0.40)	11 (0.11)			10 (0.10)	9 (0.14)	15 (0.16)	
	1 mM		52 (1.40)	40 (0.96)			18 (0.70)	18 (0.90)	60 (1.76)	

Data represent the percentage of aberrant metaphases and, in parentheses, aberrations per cell.